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# Effects of Enzymatic Activation of Bleaching Gels on Hydrogen Peroxide Degradation Rates, Bleaching Effectiveness, and Cytotoxicity

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### Clinical Relevance

The enzymatic activation of hydrogen peroxide–based bleaching agents with peroxidase optimizes tooth whitening and diminishes the diffusion of residual subproducts capable of damaging pulp tissue, which may prevent postbleaching tooth sensitivity *in vivo*.

## SUMMARY

**Objectives: The aim of this study was to evaluate the effect of horseradish peroxidase (HRP) on the release of free radicals, bleaching effectiveness, and indirect cytotoxicity of a 35% hydrogen peroxide (HP) bleaching gel.**

**Methods and Materials: First, HP degradation rates and free radical release were evaluated**

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DOI: http://doi.org/10.2341/17-276-L

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**for 35% HP in contact or not with HRP (10 mg/mL). The bleaching gel associated or not with HRP was then applied (3 × 15 minutes) to enamel/dentin discs adapted to artificial pulp chambers, and the culture medium in contact with dentin surfaces (extract) was collected and exposed to cultured odontoblast-like cells. Membrane damage and viability of cells as well as oxidative stress were evaluated. Residual HP/free radical diffusion was quantified, and bleaching effectiveness (ΔE) was assessed. Unbleached discs served as negative controls.**

**Results: The addition of HRP to the 35% HP bleaching gel enhanced the release of free radicals in comparison with plain HP gel. The 35% HP-mediated cytotoxicity significantly decreased with HRP in the bleaching gel and was associated with reduced HP/free radical diffusion through the enamel/dentin discs. ΔE values increased every bleaching session for HRP-containing gel relative to positive control, accelerating the whitening outcome.**

**Conclusion: The enzymatic activation of a 35% HP bleaching gel with HRP accelerated HP degradation mediated by intensification of free radical release. This effect optimized whitening outcome as well as minimized residual HP and free radical diffusion through enamel and dentin, decreasing the harmful effects on odontoblast-like cells.**

## INTRODUCTION

The products widely used for tooth-bleaching therapies are based on hydrogen peroxide (HP), a reactive oxygen species capable of diffusing through enamel to promote the oxidation of organic components in dentin.[1-3] Since HP features a low oxidative potential, it is believed that, to achieve effective whitening, this molecule must release highly reactive free radicals, such as hydroxyl ($HO^{\bullet}$), perihydroxyl ($HO_2^{\bullet}$), and superoxide radicals ($O_2^{\bullet}$).[4,5]

Traditionally, bleaching gels with high concentrations of HP (30%-40%) have been used for in-office therapies, aimed at accelerating the whitening effect and enhancing satisfactory esthetic outcomes.[6-11] Nevertheless, clinical studies demonstrated a high prevalence of tooth sensitivity by patients subjected to this therapy, with intensity varying from mild to intolerable.[7] It has been supposed that this negative clinical symptom may be correlated with diffusion of residual HP to the pulp chamber, causing oxidative damage to pulp cells and expression of local proinflammatory mediators.[12-14] Thus, instead of excellent esthetic effects, there is concern from a biologic point of view.[5,15]

Based on this scientific background, new alternatives have been proposed to reduce the tooth sensitivity of in-office bleaching by minimizing the residual HP capable of diffusing through enamel and dentin.[15] One interesting strategy is based on the increased HP decomposition rate into free radicals at the tooth surface, thus optimizing the reaction with tooth structure.[16-19] It is known that reaction speed and release of free radicals from HP may be influenced by transition metals that promote HP catalysis into $HO^{\bullet}$ and $HO_2^{\bullet}$ by Fenton and Fenton-like reactions.[20] However, these reactions may result in the precipitation of dark subproducts and an intense bubbling effect, reducing the stability and effectiveness of bleaching products.[16,20] Others have demonstrated that natural extracts rich in oxidative enzymes, such as catalase and peroxidase, also have the potential to enhance bleaching effectiveness by increasing the amount of free radicals but with no release of subproducts.[19,21] In biological tissues, these enzymes lead the conversion of HP into $H_2O$ and $O_2$.[22] However, Berglund and others demonstrated that horseradish peroxidase (HRP) catalyzes the degradation of HP into free radicals by activating an oxidase cycle.[23] Others have shown that peroxidase has a high affinity for peroxide, even at low concentrations and low pH, whereas catalase underwent deleterious oxidation in the presence of high concentrations of HP.[24,25]

Therefore, in the present investigation, the effect of HRP on HP degradation rates and on the free radical release of 35% HP gel was assessed, as well as its effect on bleaching effectiveness and trans-enamel/trans-dentinal cytotoxicity with pulp cells. The null hypothesis of this study was that HRP has no effect on the oxidative potential of the bleaching gel as well as on its bleaching effectiveness and cytotoxicity.

## METHODS AND MATERIALS

### HP Degradation

*Quantification of Residual HP*—For this assay, the liquid phase of a 35% HP bleaching gel (Whiteness HP 35%, FGM, Joinville, SC, Brazil) was diluted in ultra-pure distilled water (1:10; Invitrogen, Eugene, OR, USA; n=4) to obtain unsaturated measures in fluorimetry. An aliquot was transferred to tubes containing the enzyme HRP (Sigma-Aldrich, St Louis, MO, USA), yielding a final

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concentration of 10 mg HRP for each 1 mL of 35% HP solution. In the positive control, a 35% HP solution with no HRP was used (HP group). The samples were then incubated at room temperature for 0.5, 5, 10, or 15 minutes with the master mix of the Fluorimetric Hydrogen Peroxide Assay kit (1:1; Sigma-Aldrich), and fluorescence was read at 640 nm excitation and 680 nm emission (Synergy H1, BioTek, Winooski, VT, USA). The negative control group (NC), in which water instead of HP was added to the reaction, was used as the blank. The absorbance values were converted into μM of HP per mL by means of a standard curve.

*Free Radical Release*—The samples were prepared as described above (n=4). The amount of free radicals was quantified by incubation of samples with 1 mM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate probe (1:1; carboxy-$H_2$DCFDA, Invitrogen) at 37°C, and fluorescence was measured at 0.5, 5, 10, and 15 minutes (492 nm excitation and 527 nm emission; Synergy H1, BioTek).

*Hydroxyl Radical Release ($HO^{\bullet}$)*—For the specific measurement of $HO^{\bullet}$, the samples were incubated at room temperature with the fluorescent probe (1:7) of the OxiSelect Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay kit (Cell Biolabs Inc, San Diego, CA, USA), and fluorescence was measured at 0.5, 5, 10, and 15 minutes (480 nm excitation and 530 nm emission; Synergy H1, BioTek). Since the $HO^{\bullet}$ quenches the fluorescence of the probe, the amount of $HO^{\bullet}$ was calculated according to the following equation: $HO^{\bullet}$ release = fluorescence of blank (NC) − fluorescence of experimental group.

## Bleaching Effectiveness

*Enamel/Dentin Discs*—Standardized enamel/dentin discs (from 24- to 30-month-old bullocks), 5.6 mm in diameter, were obtained, and the thickness was set at 2.3 mm to simulate mandibular incisors, as described previously by de Oliveira Duque and others.[26] To achieve the desired thickness, the dentin surface was wet-ground with 400- and 600-grit silicon carbide papers (T469-SF; Norton, Saint-Gobam Abrasivos Ltda, Jundiaí, SP, Brazil) followed by treatment with 0.5-M ethylenediaminetetraacetic acid solution, pH 7.2, for 30 seconds for removal of the smear layer. The enamel surface was cleaned with a solution of pumice stone and distilled water at low speed for the elimination of superficial staining. The discs were then subjected to a staining process with black tea to standardize the baseline color as previously described,[26] and specimens with similar L* and b* values (CIE L*a*b* system) were randomly distributed among the control and experimental groups to provide standardized samples (n=8). To be read, the discs were positioned in a white silicone matrix, leaving only the enamel surface exposed. A portable ultraviolet-visible spectroscopy spectrophotometer (Color Guide 45/0, BYK-Gardner GmbH, Geretsried, BAV, Germany) was positioned over the sample, and three readings were made for calculation of the average.

*Bleaching Procedure*—The discs were distributed among the following experimental groups: NC, no treatment was performed on enamel; HP (positive control), 35% HP gel (Whiteness HP 35%, FGM), applied 3 times for 15 minutes each (3 × 15 min); and HP+HRP, 35% HP gel (Whiteness HP 35%, FGM), applied 3 × 15 minutes. For the HP+HRP group, 1 mg of HRP (Sigma-Aldrich) was incorporated into one drop of thickening agent (50 μL) by manual mixing. Then, three drops of the HP liquid phase (100 μL) were added, and the product was mixed for 15 seconds, generating a bleaching gel with 10 mg of HRP for each 1 mL of HP. This procedure was performed immediately before each application of the product. In the positive control, the same procedure was performed but with no HRP incorporation. The proportion of thickening to HP was based on the manufacturer's instructions (FGM). A 40-μL volume of bleaching gels was applied to enamel at each 15-minute application.

*Color Alteration Measurement*—After the discs were distributed among the groups, the specimens were kept in 100% humidity, with dentin and enamel in contact with cotton embedded in deionized water or saliva solution,[26] respectively, at 37°C for 72 hours to allow for similar hydration patterns among specimens. A color readout was then used to obtain baseline values for each disc. The bleaching protocol was performed on enamel surfaces, after which enamel was washed with deionized water and dried with filter paper. The specimens were incubated in 100% humidity for 72 hours to allow for rehydration, and the color readout was obtained. This procedure was repeated for six bleaching sessions. The values of L* a* b* were recorded, and the overall color change of each specimen, expressed as ΔE, was calculated according to the equation $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]½$, based on baseline values.

## Trans-Enamel and Trans-Dentinal Cytotoxicity

*Cell Culture*—The odontoblast-like MDPC-23 cells were seeded in 24- and 96-well plates (KASVI Imp.,

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Curitiba, PR, Brazil) with complete Dulbecco's Modified Eagle Medium (DMEM; supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) at 37°C and 5% $CO_2$ to obtain an 80% confluence pattern. Cells from the 56th to 60th passages were used.

*Experimental Procedure*—The enamel/dentin discs not subjected to staining were adapted to artificial pulp chambers (APC), as described previously by de Oliveira Duque and others,[26] and sterilized in ethylene oxide. The APC/disc sets were placed in 24-well plates containing 1 mL DMEM with no FBS, such that dentin was in direct contact with the culture medium and enamel remained exposed to receive the treatments according to each experimental group (NC, HP, and HP+HRP). Immediately after the bleaching procedure, the culture medium (extract) was collected and distributed into 500- and 100-μL aliquots, which were applied to the cells seeded on the 24- and 96-well plates, respectively. The cells were incubated with extracts at 37°C and 5% $CO_2$ for 1 h, after which the following experimental protocols were performed.

*Cell Viability*—The cells seeded on 96-well plates were incubated for 4 h with DMEM with no FBS, supplemented (10:1) with 5 mg/mL MTT solution (Sigma-Aldrich) at 37°C and 5% $CO_2$, after which the absorbance of formazan crystals in the viable cells was read (570 nm; Synergy H1, BioTek). The NC group was chosen as the parameter of 100% of cell viability. Therefore, the percentage of cell viability for each sample of control and experimental groups was calculated by the following equation (rule of three): Cell Viability (%) = ((absorbance of sample) × (100))/(mean absorbance of NC group); (n=10).

*Cell Morphology*—For this analysis, the cells were seeded onto round glass slides (13-mm diameter) placed at the bottoms of 24-well plates (n=3). After exposure to the extracts, the cells were fixed in 2.5% glutaraldehyde (VETEC Quimica Fina LTDA, Duque de Caxias, RJ, Brazil) for 24 hours, followed by postfixation in 1% osmium tetroxide (Sigma-Aldrich), dehydration in ethanol (30%, 50%, 70%, 95%, and 100%), chemical drying in HMDS (1,1,1,3,3,3-hexamethyldisilazane; Sigma-Aldrich), and desiccation for 72 h. The samples were mounted on metallic stubs, stored in a vacuum desiccator for seven days at room temperature, and sputter-coated with a gold layer (20-30 nm).[12-14] Cell morphology was then examined by scanning electron microscopy (5 kv; JEOL JSM 6610, JEOL Ltd, Akishima, Tokyo, Japan), at 1000 and 3000 magnification.

*Cell Membrane Damage*—This assay was performed with the Live/Dead Cell Viability/Cytotoxicity Kit (Invitrogen), which uses the fluorescence probe ethidium homodimer-1 (EthD-1), which binds to DNA bands only in cells with cell membrane rupture. The second probe was the calcein AM (CA), which is hydrolyzed by cytoplasmic esterase in cells regardless of membrane integrity. Immediately after exposure to the extracts (n=4), the 24-well plate was centrifuged (4000 rpm for two minutes) to allow detached dead cells to precipitate, the extract was carefully removed, and the cells were incubated for 15 minutes with 2 μM CA and 4 μM EthD-1. Fluorescence intensities of EthD-1 (528 nm excitation; 616 nm emission) and CA (494 nm excitation; 517 nm emission) were read (Synergy H1, BioTek), and the relative amounts of dead cells were obtained after normalization of EthD-1 with CA values. Representative images for each group were obtained by fluorescence microscopy (20×).

*Oxidative Stress*—Cells seeded on 96-well plates were pretreated with the cell-permeant fluorescence probe carboxy-$H_2$DCFDA (5 μM; Invitrogen; n=6) at 37°C and 5% $CO_2$ for 10 minutes then exposed for one hour to the extracts of each experimental group. After this period, the fluorescence intensity was monitored at 592-nm excitation and 517-nm emission (Synergy H1, BioTek), and the fold increases were calculated after normalization with the NC group.

## Diffusion of Oxidative Species and Residual HP

*Quantification of Residual HP*—A 100-μL aliquot of the extract from each group (n=6) was transferred to tubes containing 900 μL of acetate buffer solution (2 mol/L, pH 4.5), to avoid $H_2O_2$ degradation. Then, a 500-μL quantity of buffer solution plus extract was transferred to experimental tubes to react with leuko-crystal violet (0.5 mg/mL; Sigma-Aldrich) and horseradish peroxidase enzyme (1 mg/mL; Sigma-Aldrich). The final volume of reaction was adjusted to 3 mL with distilled water, and the optical density of the solutions was measured at 600-nm wavelength (Synergy H1, BioTek). A standard curve was used for conversion of the optical density obtained in the samples into μg/mL of HP.

*Free Radicals and HO•*—For these analyses, APC/disc sets (n=4) were placed in 24-well plates containing 1 mL of specific buffer supplemented

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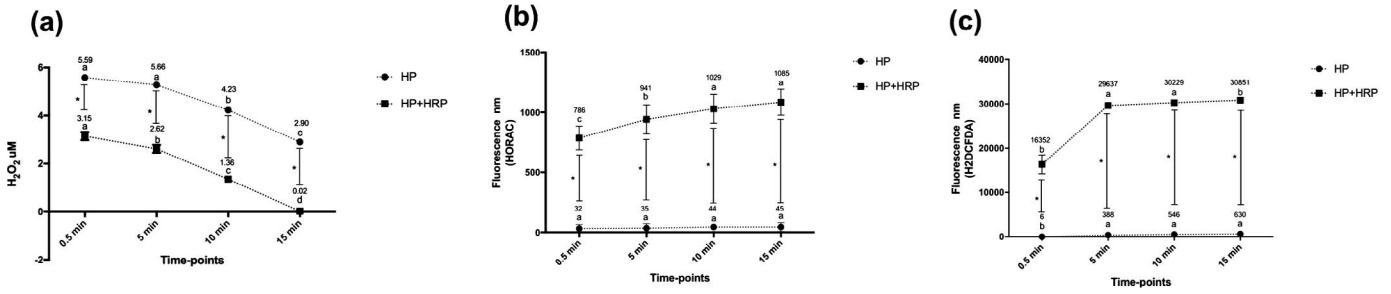


Figure 1. *Degradation kinetics of HP (a) and release of free radicals (b) and hydroxyl radicals (c) from plain HP solution (HP) and in the presence of HRP (HP+HRP) at 0.5, 5, 10, and 15 minutes. (a): Numbers are mean values of HP (μM). (b, c): Numbers are mean values of fluorescence. Different letters demonstrate significant differences among time points for each group (repeated-measures [RM] two-way ANOVA; Sidak test;* $p<0.05$*). * Significant difference among groups at each time point (RM two-way ANOVA; Tukey test;* $p<0.05$*).*

with carboxy-$H_2$DCFDA (1 μM; Invitrogen) or HORAC fluorescent probe (Cell Biolabs Inc), respectively. The specific treatments were performed on enamel surfaces, and the fluorescence intensity of the buffer was read (Synergy H1, BioTek). The NC group was used as a blank in both assays.

### Statistical Analysis

Sample size was calculated with DDS Research (Sample Size Calculator, average, two samples, α=5%; "β=95%), and a minimum of four samples per group was established for HP degradation, bleaching effectiveness, and trans-enamel and trans-dentinal cytotoxicity assays. Residual HP at 15 minutes (HP vs HP+HRP), ΔE at S1 (NC vs HP), and percentage of cell viability (MTT assay; NC vs HP) were used as parameters. Power calculation analyses were also performed by DDS Research (Statistical Power Calculator, average, two-sample, two-tail test, α=5%) at the end of the experiment, showing 100% statistical power for each evaluation. Two independent experiments were performed, and data were compiled for statistical analysis.

Data from HP degradation and bleaching effectiveness were evaluated by repeated measures two-way analysis of variance (ANOVA), complemented by Tukey or Sidak test, for the evaluation of differences among groups at each time point and the differences among time points for each group, respectively. Dunnett test was used to compare ΔE values of the HP+HRP group at each session (S) with those of the HP group at S1, S3, or S6. Numerical data from trans-enamel and trans-dentinal cytotoxicity and diffusion of residual HP/oxidative species were evaluated by one-way ANOVA complemented by Tukey test. All statistical analysis was carried out at a significance level of 5% (α=0.05).

## RESULTS

### Degradation Kinetics of Bleaching Gel

Over time, there was a significant decrease in HP concentration for both groups. A 37.6-times reduction in the amount of HP after 15 minutes was detected for the HP+HRP group relative to the HP group. A slight increase in the release of free radicals was observed for the HP group at the five-minute time point, whereas the amount of $HO^{\bullet}$ remained the same at all time points. However, an intense increase in free radicals and $HO^{\bullet}$ release was detected at all time points when HRP was added to the bleaching gel (Figure 1).

### Bleaching Effectiveness

A significant increase in ΔL as well as a decrease in Δb and Δa compared with the NC group occurred in both bleached groups at all time points (Figure 2a-c). Nevertheless, a significant increase in ΔE values was observed for HP and HP+HRP compared with the NC group (Figure 2d). Gradually enhanced ΔE throughout the bleaching sessions (S) was detected in both the HP and HP+HRP groups. For the HP group, a significant increase was detected every two sessions, whereas for the HP+HRP group, this increase was observed at all bleaching sessions up to the fifth session (Figure 2d). For determination of the effect of HRP on the speed rate of the bleaching outcome, a statistical comparison was performed between the values of ΔE at each session and those obtained from the HP group at sessions 1, 3, and 6 (Figure 2e). It was possible to detect that the bleaching effects obtained after one and two sessions in the HP+HRP group were statistically similar to those obtained by the HP group at the third and sixth sessions, respectively. A statistically significant difference was also detected between the HP

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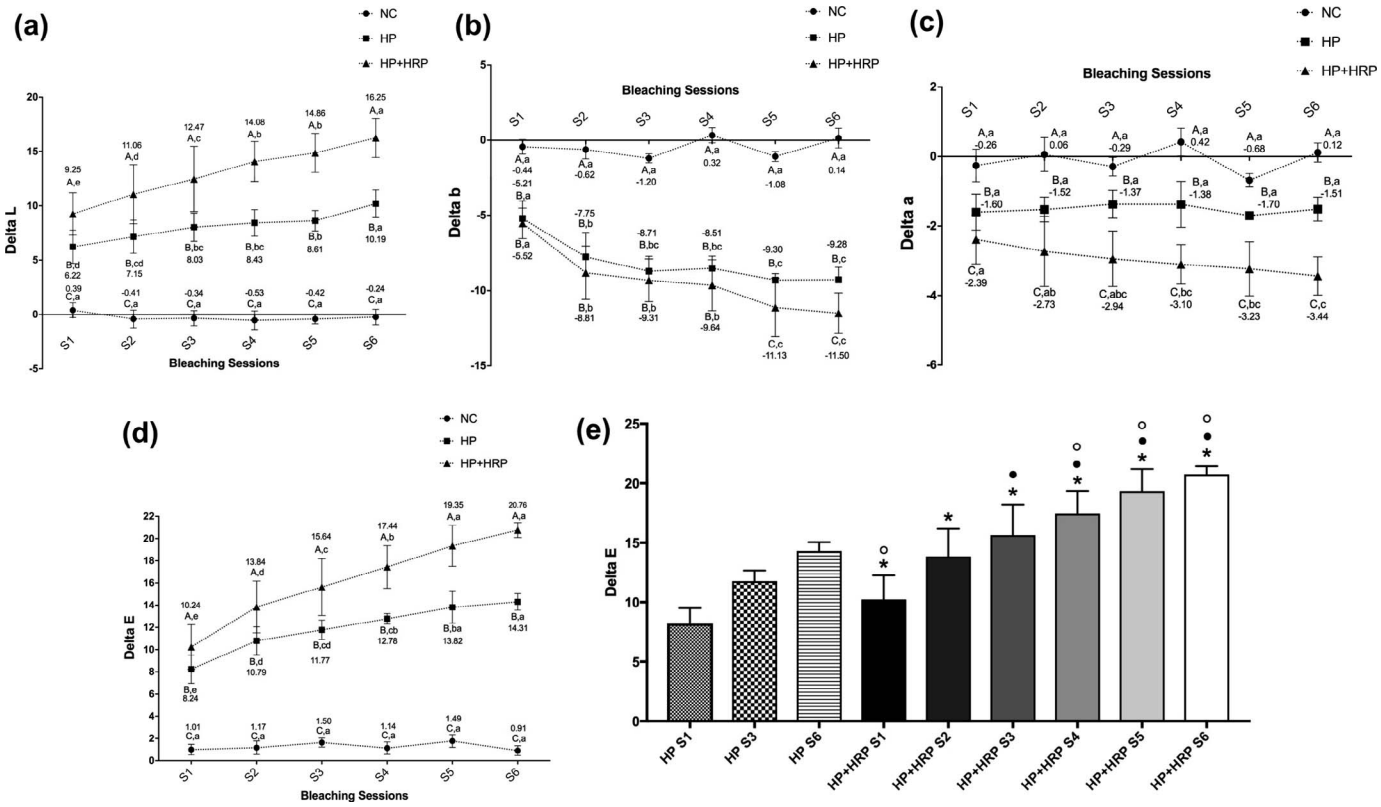


Figure 2. *(a-d): ΔL, Δa, Δb, and ΔE values for each group throughout the bleaching sessions (S1 to S6). Numbers are mean Δ values. Lowercase letters allow for comparison among time points for each group (repeated-measures [RM] two-way ANOVA; Sidak test). Uppercase letters allow for comparison among groups at each time point (RM two-way ANOVA; Tukey test). Different letters demonstrate significant differences (*$p<0.05$*). (b): Statistical comparison between ΔE values for the HP+HRP group at each bleaching session (S) with ΔE values of the HP group at S1, S3, or S6 (one-way ANOVA; Dunnett test). * Significantly different from the HP group at S1. • Significantly different from the HP group at S3. ° Significantly different from the HP group at S6 (*$p<0.05$*).*

group at session 6 and the HP+HRP group at session 4.

### Trans-enamel and Trans-dentinal Cytotoxicity

A significant reduction in cell viability was observed at around 75.9% and 42.4% for the HP and HP+HRP groups, respectively, in comparison with the NC group. However, the HP+HRP group featured cell viability values significantly higher than those of the HP group (Figure 3a). These data were correlated with scanning electron microscope images, where alterations were observed in the morphology and numbers of cells adhering to the glass surfaces of bleached groups relative to those of the NC group, which were slightly less intense in the HP+HRP group (Figure 3d). The live/dead and oxidative stress assays demonstrated significant increases in positively stained cells in the bleached groups compared with those in the NC group; however, this staining was significantly less intense in the HP+HRP group compared with the HP group (Figure 3b-e).

### Diffusion of Bleaching Gels' Subproducts Through Enamel and Dentin

A significant reduction in the trans-enamel and trans-dentinal diffusion of HP and free radicals was detected in the presence of HRP (Figure 4a and b). $HO^{\bullet}$ was found to be capable of diffusing through the discs, with numerical values being higher in the HP+HRP group in comparison with the HP group; however, no statistically significant difference was detected (Figure 4c).

## DISCUSSION

The reduction of HP diffusion through dental structure is considered a hallmark for the development of biocompatible bleaching products, especially in teeth with thin enamel/dentin substrate, such as incisors, which are susceptible to the adverse effects of in-office bleaching therapies.[15,27] Aiming to increase bleaching gel reactivity with tooth structure and to minimize the residual HP diffusion to pulp chamber, the present investigation incorporated the

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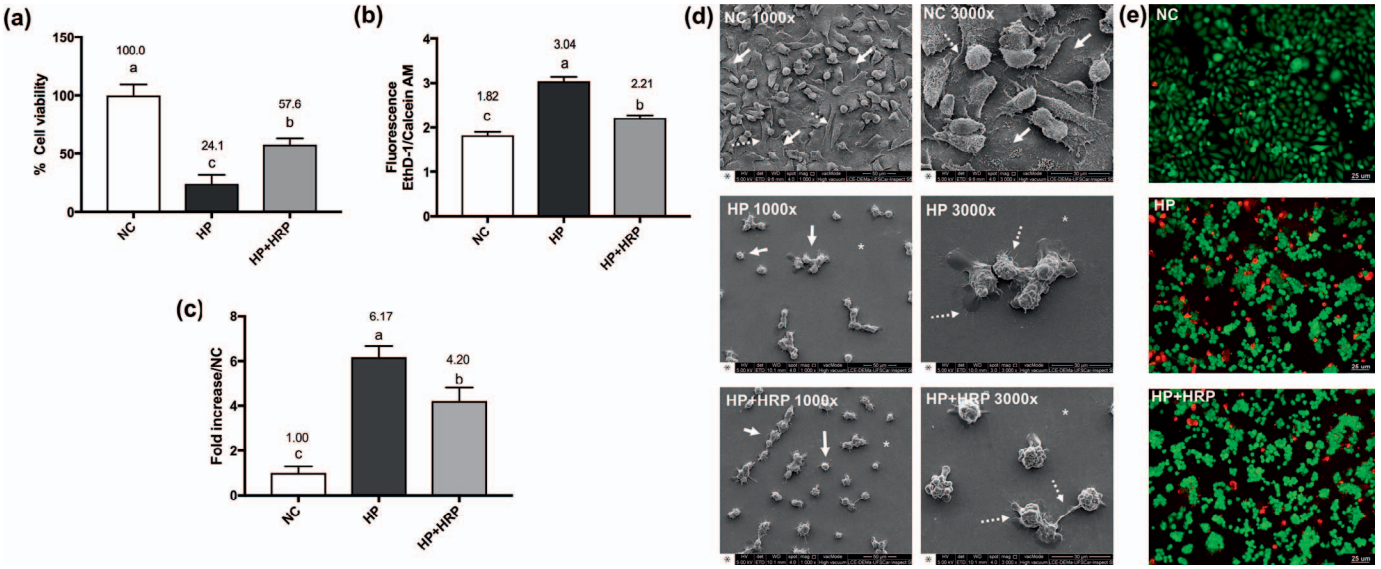


Figure 3. *Bar graph of the MTT assay (a) and oxidative stress (b) and cell membrane damage (c) assays. Numbers are mean values, and different letters demonstrate significant differences among groups (one-way ANOVA; Tukey test;* $p<0.05$*). (d): Representative scanning electron microscopy images. Note that the NC group features a large number of cells with a polygonal shape covering the entire glass surface (solid arrow). Several long cytoplasmic projections can be observed (dashed arrow). In the HP and HP+HRP groups, large areas of glass substrate (*) can be observed, demonstrating a reduction in the number of cells adhering after exposure to extracts. The adherent cells featured reductions in cytoplasmic spread and alterations of surface morphology (solid arrow). Thin cytoplasmic projections can be observed attaching cells to the glass surface (dashed arrow). (e): Representative images of live/dead fluorescence staining with EthD-1 (red) and CA (green) for each experimental group.*

horseradish peroxidase (HRP) enzyme into a commercial 35% HP bleaching gel. According to the results, the null hypothesis was rejected, since the incorporated HRP played a significant role in all the parameters tested in this experiment.

The degradation of HP may release different subproducts, depending on the environmental conditions. In an aqueous neutral medium, the following reaction occurs: $H_2O_2 \rightarrow H_2O + O_2$. In the presence of a substrate, such as a chromogen, the reaction $H_2O_2 + S \rightarrow S + HO^{\bullet} + HO^{-}$ (S = substrate) takes place.[22] Therefore, the reduction of HP concentration through time in the HP group may be mostly a consequence $H_2O$ and $O_2$ release, since only a slight increase in free radical ($H_2$DCFDA probe) was observed and no chromogen was added to the reaction. Indeed, the absence of chromogens in these reactions allowed us to demonstrate that HRP induces degradation of HP into free radicals. According to our results, in the presence of HRP, there was acceleration in the HP degradation rate as well as an intense release of free radicals, including $HO^{\bullet}$, in comparison with the plain 35% HP solution. This effect may be related to the catalytic cycle mediated by HRP in the presence of HP, which results in the formation of several highly oxidative intermediates with unpaired electrons on the outer layer, such as described previously by Berglund and others and Veitch. According to those authors, HRP in a resting state has a ferric center that may be transformed into several oxidative species when in contact with HP, leading to the release of $HO_2^{\bullet}$ species.[23,28] Alternatively, the ferric center may act as a substrate for the Fenton reaction, with release of $HO^{\bullet}$ according to the following reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$.[17] Based on the results obtained in the present investigation, we suggest that the presence of HRP in the 35% HP bleaching gel caused the formation of highly oxidative intermediates involved in the catalytic cycle, as described

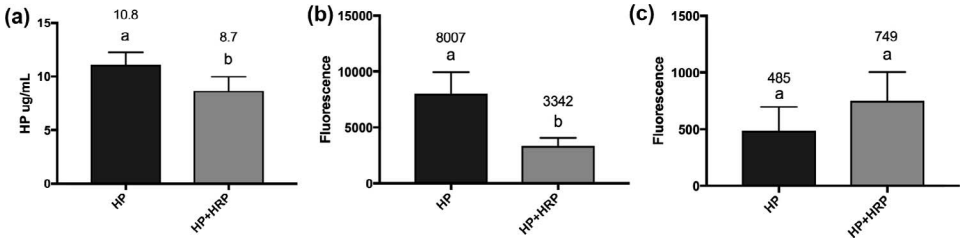


Figure 4. *Trans-enamel/trans-dentinal diffusion of HP (a), free radicals (b), and hydroxyl radicals (c). Numbers are mean values, and different letters demonstrate significant differences among groups (Student t-test;* $p<0.05$*).*

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above, with release of free radicals, such as $HO_2^{\bullet}$ and $HO^{\bullet}$.

For verification of the increased oxidative potential of HRP-containing gels, the dental color alteration was measured throughout several bleaching sessions. This assay demonstrated the intense increase of bleaching effectiveness in the presence of HRP compared with that of the gel without this enzyme. As previously observed, the 35% HP gel caused intense color alteration after the first bleaching session; however, significant color alteration over time was detected at the interval of two bleaching sessions. In contrast, a significant increase in ΔE values at each session was detected for the HP+HRP group, up to the fifth session. Overall, increases of about 24.3%, 28.3%, 32.9%, 36.5%, 40.0%, and 29.7% in ΔE values from the first to the sixth sessions were detected for the HP+HRP group relative to the HP group. In addition, there was an acceleration of bleaching outcome in the presence of HRP, since the same bleaching pattern obtained after three and six sessions for traditional plain 35% HP gel was reached after only one and two sessions, respectively, for the gel associated with HRP. This enzyme also increased the bleaching power over time; from the fourth session, the ΔE values were significantly higher than those observed for the traditional gel at the sixth session. Therefore, the intense oxidative power of HP bleaching gels in the presence of HRP was demonstrated. Gopinath and others demonstrated that 10% and 35% HP gels reached the same bleaching outcome in the presence of a natural extract rich in catalase and peroxidase, regardless of the HP concentration in the product.[21] Travassos and others observed a 42.1% increase in bleaching effectiveness (ΔE) for a 35% HP gel after one single $3 \times 10$-minute session when a peroxidase-rich extract was incorporated into the gel.[17] The authors speculated that this positive effect was related to an increase in $HO^{\bullet}$ formation mediated by peroxidase.[17,21] The release of high amounts of $HO^{\bullet}$ in the presence of HRP was demonstrated in the present investigation, compared with the limited amount released by the plain 35% HP gel. It is suggested that the release of $HO^{\bullet}$ may have played the major role in the increased oxidative power of bleaching gel in the presence of HRP, since its oxidative potential was about 1.6 times greater than that of HP and $HO_2^{\bullet}$.[20]

In the present study, besides the positive effect on bleaching effectiveness, the bleaching gel containing HRP also reduced the residual HP diffusion through enamel/dentin discs. Torres and others[18] reported that the addition of a chemical activator capable of accelerating the decomposition rate of HP into free radicals to bleaching gels reduced the diffusion of HP throughout tooth structure. Therefore, it is believed that these substances directly react with HP or play a role in the decomposition reaction, accelerating the release of free radicals and diminishing the diffusion of free HP to the pulp chamber.[16] In the present investigation, we also detected that, instead of HRP-containing gel releasing high concentrations of free radicals at tooth surfaces, a lower amount of such harmful agents was capable of diffusing through enamel and dentin compared with the positive control. We suggest that these free radicals interacted with tooth structures before reaching deep dentin, mainly because of their extremely short half-life ($10^{-9}$ s).[20] In contrast, the free radicals detected in plain 35% HP gel may be a consequence of HP degradation in deep dentin.

In terms of the biologic effects, it was also demonstrated that HRP significantly minimized the trans-enamel/trans-dentinal damage mediated by 35% HP gel on odontoblast-like cells *in vitro*. The oxidative stress was reduced by about 32% in those cells exposed to the extracts of HRP-containing gel compared with a positive control, resulting in cell viability values around 39% higher. It is important to note that, in the present study, 2.3-mm-thick discs simulating mandibular incisors were used to test the positive effect of HRP in a critical situation. Histopathological *in vivo* studies have already demonstrated that human mandibular incisors are the teeth most susceptible to pulp damage, whereas premolars may suffer only slight damage when subjected to 35% HP gels.[27,29-31] De Oliveira Duque and others showed that the trans-enamel/trans-dentinal extract obtained from a 35% HP gel applied to 4.0-mm-thick discs (simulating premolars) was about 56% less damage to cultured human dental pulp cells than that obtained from 2.3-mm-thick discs.[26] Nevertheless, it is important to state that the 35% HP gel applied 3 times for 15 minutes each, associated or not with chemical activators, promoted significant negative effects on the odontoblast-like cells, mediated by oxidative stress followed by cell membrane damage. The establishment of an inflammatory reaction in human mandibular incisors following this same bleaching therapy has been correlated with the clinical symptom of tooth sensitivity.[27,29-31]

In the present study, the odontoblast-like cell lineage was selected since it features phenotypes similar to those of human odontoblasts, which are

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the first cells to come into contact with the products leached from bleaching gels capable of reaching the pulp chamber.[15] However, previous studies have demonstrated that this cell lineage is more resistant to the oxidative stress mediated by HP arising from bleaching gels than is the primary culture of human dental pulp cells.[16,32] Thus, despite the exciting data obtained in this study, we should consider that the addition of chemical/enzymatic activators to bleaching agents with high concentrations of HP may still cause harmful effects to pulp cells. On the other hand, the results obtained from laboratory studies may be considered overestimated, since a monolayer cell culture was established and other *in vivo* conditions, such as extracellular matrix, host immune cells, and intrapulpal pressure, may play a significant role on the adverse effects of bleaching therapies in the clinical situation.[15] Another important limitation of this study is that nonstained discs were used for the biological assays. As demonstrated by Moreira and others, the intensity of dental pigmentation plays a significant role in HP diffusion through enamel and dentin.[33] The lower the L* value, the lower the HP diffusion. Since free radicals released from the bleaching gel should interact with the chromophores found in dentin, the absence of stains in the enamel/dentin discs used in the biological assay potentially allowed a more intense diffusion of nonreacted products from the gel.

Therefore, based on the limitations of this *in vitro* study, further experiments are needed to better understand the effects of HRP-activated HP gels with tooth structure as well as to determine the ideal HP-to-HRP proportion capable of promoting effective bleaching with no damage to pulp cells.

## CONCLUSION

It was concluded that the enzymatic activation of a 35% HP bleaching gel with HRP accelerates HP degradation rates mediated by intensification of free radical release. This positive effect optimizes and accelerates the whitening outcome as well as minimizes the diffusion of residual HP and free radicals through enamel and dentin, decreasing the harmful effects of oxidative agents on odontoblast-like cells.

### Acknowledgements

This work was supported by the São Paulo Research Foundation–FAPESP (grants 2015/21770-4 and 2016/15674-5), the National Council for Scientific and Technological Development–CNPq (grants 303599/2014-6 and 442336/2014-4), and Coordination for the Improvement of Higher Education Personnel–CAPES (grant 1587404).

### Conflict of Interest

The authors of this article certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

*(Accepted 8 June 2018)*

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